The Journal of Research on the Lepidoptera 24(4):334-358, 1985(86)

Invited Paper

Enzyme Electrophoresis and Interspecific Hybridization in Pieridae (Lepidoptera)

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Abstract. Sterility and incompatibility levels found in crosses of 12 Lepidoptera species (family Pieridae) were compared with differences between electrophoretic enzymatic patterns characteristic of the respective species (Geiger, 1978, 1981). Good agreement was found when different subgenera and genera were crossed while considerable disagreement prevailed at the subspecies and species levels. Inadequate estimates of low level taxa divergency, uneven development of reproductive isolating mechanisms and limitations of the scope of the electrophoretic approach are discussed as possible reasons for the observed discrepancies.

Key words: enzyme electrophoresis, hybrid sterility, interspecific crossings, taxonomical levels, Pieridae, Lepidoptera.

Introduction

Electrophoretic studies of enzymatic similarity are used increasingly to trace genetic relationships among taxa and to make phylogenetic interpretations. Indeed, Bullini (1983) referred to the technique as a revolution in taxonomy, or at the least a transformation phase of this discipline leading to a better comprehension of taxonomy as well as evolutionary processes. In the Lepidoptera, studies of populations, subspecies, sibling species and species complexes, and whole families have already been made, an oversight of Bullini (1983). Using diurnal Lepidoptera, Geiger (1978, 1981, 1984) carried out an extensive study on variations of 20 isoenzymes in 24 European species representing four subfamilies of the family Pieridae, and found good agreement between the coefficient of their enzymatic similarity, Nei's I value, and their systematic position and phylogenetic relationships.

Hybridization among 13 of the 24 Pierid species studied by Geiger were carried out at the biological laboratory in Zagreb for many years (Lorković, 1928, 1969, 1978). In combination with Geiger's data, this largely unpublished work has acquired new significance. Therefore some selected data will be presented here. The degree of relationship among the 12 *Pieris* species emerging from these hybridization experiments will be

compared with those derived from the enzyme studies of Geiger. A list of the primary works on enzyme genetics by Ayala, Nei, Lewontin and other authorities can be found referenced in the papers cited herein. No general discussion is made here of views on speciation, microevolution and phylogeny unless they are directly related to the subject in question.

Methods and Material

a) Crossing methods

Crosses were made using laboratory bred specimens derived from wild-inseminated females. Males were occasionally replaced with wild specimens to avoid inbreeding depression (see Oliver, 1981).

As the studied taxa, with two exceptions, were sexually isolated, interspecific crossings would naturally occur only exceptionally. Therefore, three artificial original crossing techniques were necessary:

(1) **natural pairing,** in which the pair designed for crossing was placed in a glass vessel together with an inactive male belonging to the species of the female. Pheromones released by this male could stimulate the female to accept the foreign male. In Pieridae, as with most other butterflies, it is usually the female which is able to distinguish between homospecific and heterospecific males, so that by such method mating may take place quickly without intervention from the breeder.

(2) Since Pieridae are too delicate for the rather rough hand-pairing, reintroduced by Clarke (1956), the **forceps-hand pairing** method (Lorković, 1947, 1953) was used where applicable. In *Colias*, the uncus and valvae of the male are too short for manipulation, even with the finest of forceps, thus making this method impracticable.

(3) Therefore the original **gynanaesthetic method** (Lorković in Friedrich, 1975, 1982, 1983) was used, in which the anaesthetized female is held with a pair of forceps by her folded wings and presented to the male in a cage. The male then grasps the genital part of the female abdomen with his uncus and valvae.

Rearing took place in the laboratory of the Biological Institute in Zagreb, in a room on the sunny side, with temperatures between 20°C and 30°C in summer with 70% R.H. and 50% R.H. in winter. Egg-laying and rearing of the young larvae, up to the 4th instar, took place on potted plants. Later food plants with stems in water were used. The cages employed were 25 cm cubes covered with fine nylon.

b) Material, Species & Sources

Species	Origin
Pieris rapae (Linneus, 1758)	Zagreb, Croatia, Yugoslavia
P. mannii (Mayer, 1851)	Adriatic coast, Plitvicka jezera, Zagreb (Croatia), St. Martin Vesubie (France, 1936)
P. ergane (Geyer, 1828)	Adriatic coast, Zagreb, Julian Alps

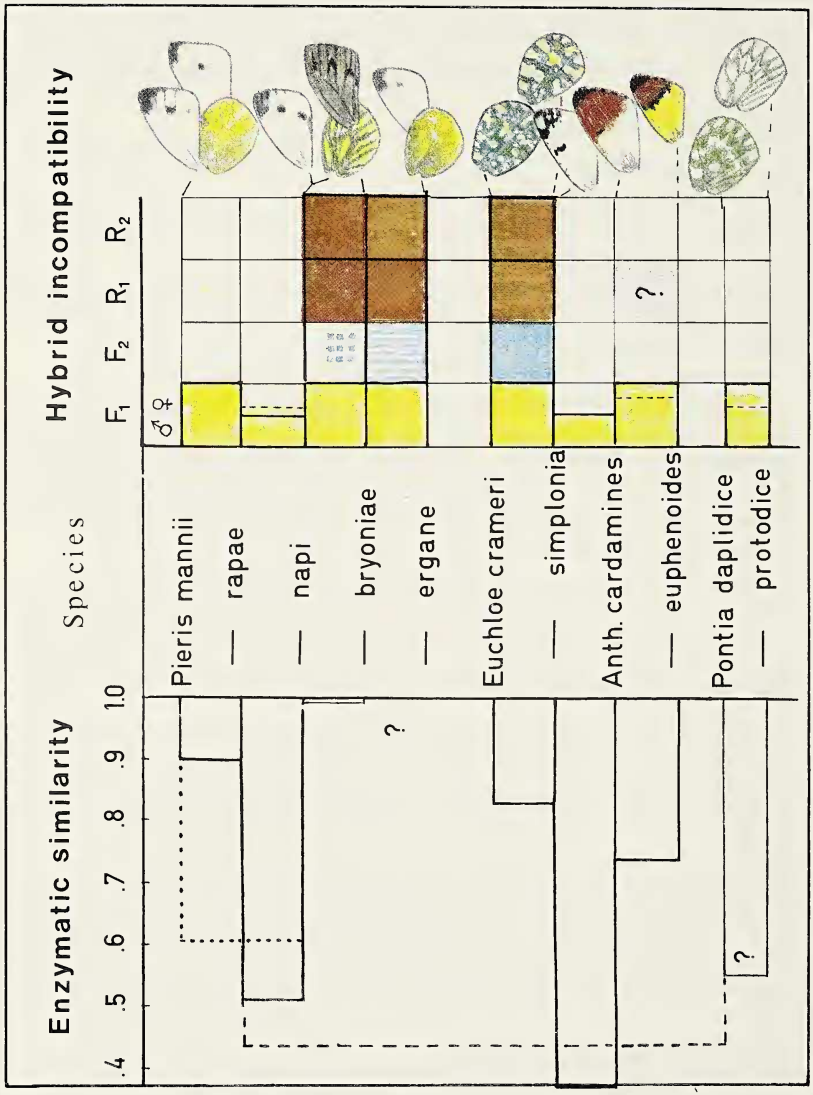

Enzymatic similarity
.4 .5 .6 .7 .8 .9 1.0
Species
Hybrid incompatibility
♂♀ F1 F2 R1 R2
Pieris mannii
— rapae
— napi
— bryoniae
— ergane
Euchloe crameri
— simplonia
Anth. cardamines
— euphenoides
Pontia daplidice
— protodice
?
?
?

Plate I.

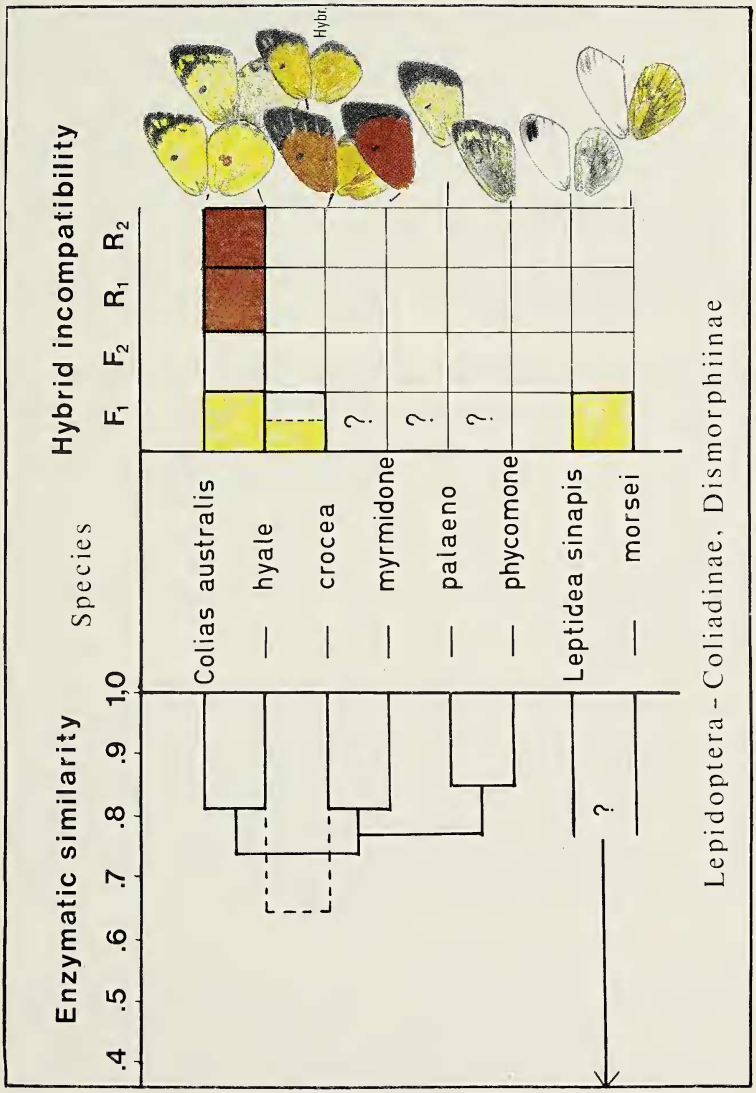

Enzymatic similarity
.4 .5 .6 .7 .8 .9 1,0
Species
Hybrid incompatibility
F1 F2 R1 R2
Colias australis
— hyale
— crocea
— myrmidone
— palaeno
— phycomone
Leptidea sinapis
— morsei
?
? ? ?
Hybr.
Lepidoptera - Coliadinae, Dismorphiinae

Plate II.

P. napi (Linneus, 1758)	Zagreb, Adriatic coast, Julian Alps; England; Cantabria Mts., Sierra de Gredos (Spain, 1972, Eitschberger)
P. bryoniae (Linneus, 1758)	Slovenian Alps, Austrian Alps, Bad Vöslau (Vienna); Les Mées, Les Fonds (Basses Alpes) (Descimon); Zermatt (Penninian Alps, 1964)
P. (pseudorapae) balcana (Lorković, 1968)	Treska gorge, Negorci (Macedonia), Zelengora (SE Bosnia), Slunj, Plitvicka jezera (Croatia)
P. oleracea (Harris, 1829)	New Hampshire, USA (S. R. Bowden)
Pontia daplidice (Linneus, 1758)	Zagreb
P. protodice (Boisduval, 18)	Chicago (USA)
Euchloe simplonia (Boisduval, 1828, nec **simplonia** Freyer, 1829)	Zermatt (Switzerland, 1964; W. Back, 1979)
E. ausonia graeca (Staudinger, 1869)	Dalmatia (Croatia), Treska gorge (Macedonia)
E. crameri (Butler, 1869)	Nice (France, 1936); Les Mées (Basses Alps), W. Back, 1979
Anthocharis cardamines (Linneus, 1758)	Zagreb, Podsused, Pustodol
A. euphenoides (Staudinger, 1869)	Nice (France, 1936)
C. alfacariensis Berger, 1948, (=**australis** Verity, 1911)	Zagreb, Ika; Véseley (Dijon, France)
C. crocea (Geoffroy in Fourcroy, 1785)	Zagreb

Additonal information was also obtained from crossings of the European *Pieris napi* and the Japanese *P. melete* (Ménétriés, 1857), *P. nesis* Fruhstorfer, 1908 (=*japonica* Shirozu, 1952) and *P. dulcinea pseudonapi* Verity, 1911; of *P. rapae rapae* L. with *P. rapae crucivora* (Boisduval, 1836); and of *P. marginalis* (Scudder, 1861), *P. virginiensis* (Edwards, 1870) and *P. oleracea* (Harris, 1829) from North America, the latter kindly supplied by S. R. Bowden. The data on these crosses are not included in the present paper, except those involving *P. oleracea*, because of the lack of correlative enzymatic data.

A survey of common features involving interspecific hybrids

The purpose of this work is to compare two criteria for Pierid genetic relationships. One of these, enzymatic, is relatively straightforward and quantifiable while the other, sterility, is complex. As a measure of genetic incompatibility, sterility is a consequence of a number of developmental barriers contributing unequally to the ultimate failure of hybrid reproduction. The term "sterility" is used here in the broadest sense to denote incapacity of reproduction irrespective of the exact mechanism of hybrid inviability (e.g., failure of gametogenesis, zygote lethality, loss of fecundity, etc.). A "sterility" grading system was therefore established to score

for developmental success. Usefulness of the system requires a knowledge of the degree of incomplete development. Some typical hybrid characteristics are described below.

First, it will be recalled that in Lepidoptera disturbance of fertility generally occurs more frequently in females than in males because the females are the heterogametic sex (i.e., having a pair of unequal sex-determining chromosomes, XY or XO) which, in agreement with Haldane's rule, renders the females more affected than males in crosses. The reader can refer to the following works for further discussion on the properties of sterility in interspecific hybridization: Bytinski-Salz (1930, 1934), Federley (1911-1953, for bibliography see Suomalainen, 1952), Clarke and Sheppard (1953, 1955, 1964, etc.), Remington (1956, 1958, 1960, 1968), Bowden (1956) and most recently Oliver (1977, 1979a, b) (for bibliography see Suomalainen, 1952).

1. The least obvious genetic disturbance is a shortened development time of hybrid females, known as protogyny (eclosing of females before males). Protogyny does not involve the sterility of an individual hybrid. It is merely a disadvantage to the population as a whole, but may however favor back crosses.
2. A more serious sterility producing effect encountered in females is a physiological disturbance of diapausing female larvae or pupae consisting in their inability to terminate their diapause period. This leads to a lack of viable females in the spring brood and results in abnormal sex ratios in the adult population. When females develop directly (without diapause) they are fertile at least to a degree.
3. Further in the descending order of fertility, female sterility is encountered preventing the appearance of an F_2. The near absence of the F_2 generation is the main characteristic of the hybrids studied here, excepting hybrids in the genus *Euchloe*. At this level of sterility, reproduction is limited to the male sex in backcrosses (R_1).
4. At the fourth level, both sexes are sterile in the F_1 and the females show deformation, usually expressed as crippled wings, while the males are more viable and better developed.
5. The lowest fertility was found in intergeneric matings such as *Pontia* x *Pieris* which are characterized by a high egg-laying rate and egg fertility while embryonic development is 95% incomplete. With this case, the fertility limit for Lepidoptera appears to be reached.

Data on egg laying, egg embryonic development and rate of larval hatchability are given here only for the last mentioned crosses of *Pontia* x *Pieris*. In the other crosses the role played by the egg development is reflected in part in the numbers and the degree of sterility of F_1, F_2 and R generations. A more thorough analyses is beyond the scope of this paper.

Plates I and II summarize relationships between the taxa discussed here based on comparison of enzyme electrophoresis and hybrid sterility analysis. The left hand column presents dendrograms based on enzymatic similarity, $\bar{I}$ (Geiger, 1981). In the remainder of this paper enzyme relationships are expressed as difference (EDf) in order to directly compare values with sterility grades. The right column gives sterility values for F_1, F_2, and two backcross (R_1 and R_2) crosses. A full colored square denotes normal development of adult males and females. Absence or numerical deficiency of either sex (usually the female) is indicated by a line dividing the square into two halves, one less colored than the other. When the F_2 is represented by a few individuals, the respective square contains a few dots or stripes.

At the right margin of Plates I and II one parental fore- and/or hindwing of the species involved in the cross mating is given. These figures show the morphological distinction between the species crossed. For the cross *Colias crocea* x *C. hyale* a hybrid male is also presented. For the crosses *Pieris bryoniae* x *P. ergane* and *Leptidea morsei* x *L. sinapsis* enzyme data are not available, while three of the *Colias* have not yet been crossed.

Sterility Grades

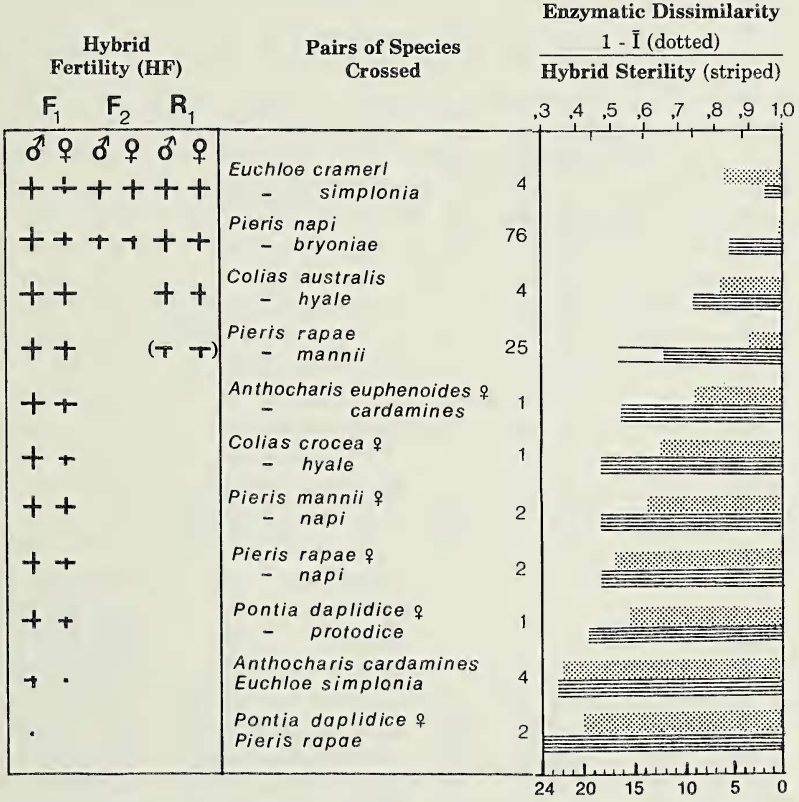
By comparing the enumerated properties of hybrids, it is possible to arrange the hybrids in a series of decreasing fertility (or increasing sterility). Exact grading is not possible because the differences between several crosses were minimal or undetectable. In Table 1, left column, the presence of completely developed adult males and females in F_1, F_2 and R_1 is indicated by a cross, +. any departure from normal development is indicated by a reduced length of the respective arms of the cross: the left arm = size, the upper arm = number of offspring, right arm = development (crippling) and the lower arm = inviability. Lower numbers of one sex are identical with skewed sex ratio, a variable therefore not separately identified. Inviability is in part involved in the first three characters, while in the fourth arm the viability of the adults is to be understood.

Indices of hybrid sterility in Table 1 were derived by multiplying relative arm lengths with a full arm receiving a value of one, a case of complete fertility of both sexes for the three test generations would be 4 (full arms) x 2 (sexes) x 3 (generations) = 24. Total sterility would be 0. The values obtained are given in detail in Table 2.

Comparison of Crosses and Enzymatic Data

Enzyme dissimilarity (EDf), $1-\bar{I}$, values are employed in the following rather than the enzyme identity ($\bar{I}$) values used by Geiger (1981) in the construction of dendrograms illustrating taxonomic affinities in the

Table 1. Comparison of hybrid fertility (HF, left), hybrid sterility (HS) and enzymatic dissimilarity (EDf) values (right) (Geiger, 1982) in 13 taxa of European Pieridae. Degrees of hybrid sterility are based on properties of reporduction in the F_1, F_2 and R_1 generations.



Key to symbols:

+ normal

⊦ undersized

⊤ lower numbers

⊣ crippled

⊥ inviabilized

• no larval hatch

The relative length of an arm corresponds to the degree of effect.

Table 2. The degree of hybrid fertility of both sexes in F_1, F_2 and R_1 generations and the total hybrid fertility (HF) versus hybrid sterility (HS) in the interspecific crosses in Pieridae.

	Taxa Crossed	F_1		F_2		R_1			(HF)	(HS)
	Female Male	♂	♀	♂	♀	♂	♀		♂ + ♀	♂ + ♀
1.	**E. crameri x E. simplonia**	4.00	3.00	3.75	3.75	3.90	3.75	=	22.15	1.85
2.	**P. napi x P. bryoniae**	4.00	2.25	2.25	1.75	3.75	3.50	=	17.60	6.40
3.	**C. hyale x C. australis**	4.00	4.00	—	—	3.50	3.30	=	14.80	9.20
4.	**P. rapae x P. mannii**	4.00	3.25	—	—	(2.25	2.75)	=	12.25 (7.57)	11.75* (16.43)
5.	**A. euphenoides x A. cardamines**	4.00	2.75	—	—	—	? —	=	7.75	16.25
6.	**C. crocea x C. hyale**	3.80	1.95	—	—	—	—	=	5.75	16.25
7.	**P. rapae x P. napi**	3.00	2.75	—	—	—	—	=	5.75	18.25
8.	**P. mannii x P. napi**	3.50	2.05	—	—	—	—	=	5.55	18.45
9.	**P. protodice x P. daplidice**	3.00	1.10	—	—	—	—	=	4.10	19.90
10.	**E. simplonia x A. cardamines**	1.60	0.05	—	—	—	—	=	1.65	22.35
11.	**P. daplidice x P. rapae**	0.04	—	—	—	—	—	=	0.04	23.96

*Although in *rapae* x *mannii* the number of backcross matings achieved and eggs laid is 3.5 times greater than in *Euchloe* and *Colias* crosses, the number of adult offspring is 6.6 times smaller. Therefore, the sterility grade of R_1 (*rapae* x *mannii*) must be reduced from 5.00 (= 2.25 + 2.75) to 5.00 : (3.5 x 6.6) = 0.22.

Pieridae. It seemed appropriate to present his data as EDf values for comparison. The corresponding way to present the hybridization data was to use the hybrid sterility (HS) scale. In Table 1 (right hand column) the 0.3 - 1.0 portion of the EDf scale was arbitrarily juxtaposed to the whole (0 - 24) HS scale, because enzymatic I values below 0.3 were the lowest limit of enzymatic similarity that Geiger found.

Results

The comparative quantitative data are given in Table 2. The striking feature here is the high hybrid sterility (HS) values of crosses 4 - 11, which are related to the failure of F_2 and R_1. HS values are less in crosses 1 - 3. The extreme HS values found in crosses 10 and 11 reflect the fact that these resulted from mating individuals belonging to species of different genera. Although the crosses were arranged according to the rising HS values, the enzymatic dissimilarity (EDf) of the hybrids increases more uniformly than the HS values. However, considerable discrepancies exist between the HS and EDf values in crosses 1 - 4, all of which are taxa belonging to the lowest taxonomic level. In cross 1, HS is less than EDf, while in cross 3 HS is .3 greater than EDf, while in cross 2 HS is 31 times greater than EDf. Another discrepancy is found in cross 4 in which HS is more than 3 times larger than EDf.

The discrepancies between the HS and EDf values deserve further analysis. The pair *Pieris rapae* x *P. mannii* (cross 4) will be considered first. The two species here represent a case of full biological speciation in spite of such similarity of external appearances that the two species leads sometime to their false identification.

Pieris rapae - P. mannii. These two species are reproductively completely isolated, having an index of sexual isolation approaching unity in full sympatry, although not always syntopically (unpublished data). In spite of generalized morphological similarity, these species differ in at least 24 morphological characters found at all developmental stages, from egg to adult. In 19 crosses, normally developed individuals of both sexes occurred only when the mother was *rapae*, while with *mannii* mothers the females were missing with few exceptions (8%). The F_2 is completely blocked in both directions because the females in F_1 contain undeveloped eggs, usually producing no eggs at all (Figs. 1 and 2). Although the chromosome numbers are the same in both species (N = 25), chromosome pairing during meiosis of the spermatocytes (Fig. 3) is so highly disturbed that the fecundity of the males is 7 to 1000. From 32 back-crosses with females of both species in 6 of the 8 possible mating combinations 1,310 eggs were laid, but not more than 9 larvae hatched and 8 males and 1 female eclosed (0.68%). Three males were slightly crippled. No eggs were produced either from two F_1 x F_1 matings or from one R_2 backcross. It is

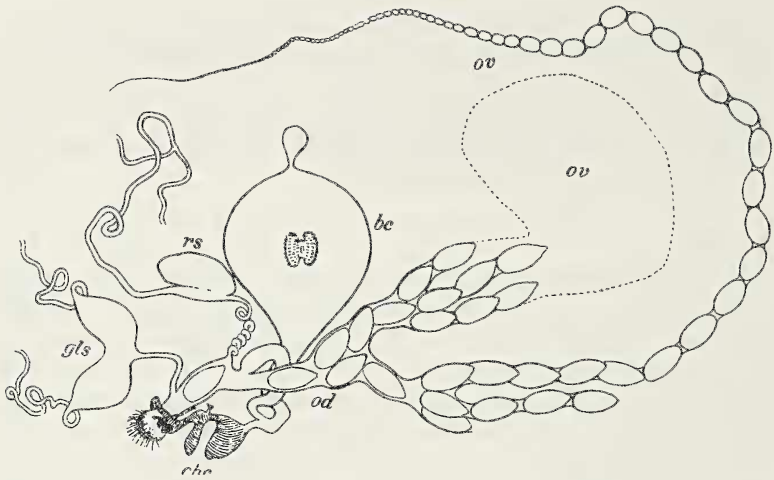


Fig. 1. Internal and external female genitalia of *Pieris rapae* L. with normally developed ovaries of which only one ovariole is spread. (after Lorkovic', 1928).

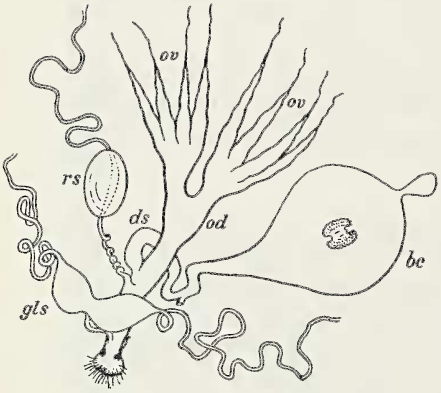


Fig. 2. Female genitalia of an adult *Pieris rapae* x *P. mannii* hybrid with short ovarioles lacking eggs entirely. Note the other fully developed genital organs (the external genitalia removed). (after Lorkovic', 1928).

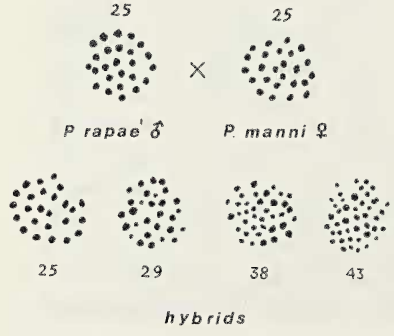


Fig. 3. Metaphase chromosome plates of the first meiotic division in spermatogenesis of *Pieris rapae* x *P. mannii* with 25 bivalent chromosome (upper row) and their F_1 hybrids with different chromosome pairing, from normal 25 to 43, with only 6 chromosome pairing. Slides No. 0143, and 0139 (1934).

evident from these data that *P. rapae* and *P. mannii* are two reproductively incompatible taxa and that they cannot be regarded as anything but two full species, bearing all typical species properties. Nevertheless, the EDf value for these two species is 0.10, much less than the minimum of 0.20 - 0.30 suggested for such cases by Bullini, et.al. (1981) and nearly half that of the next two taxa.

Euchloe crameri - *E. simplonia*. These two species are clearly less genetically differentiated than the above *Pieris* species, yet their EDF value is 0.17, much larger than the value 0.10 for the two *Pieris*. It is worth recalling that until recently these two taxa were considered subspecies of *E. ausonia*, because of their morphological similarity and with separate distribution ranges. In fact, the nomenclature problem of this complex is far from satisfactorily resolved (Back, 1979). Since the results of many crosses are not yet available (Lorković and Back, in prep.), only corsses of *E. crameri* and *E. ausonia graeca* from Dalmatia and the Balkans will be considered here. *E. ausonia graeca* is very close to *E. simplonia*. There are neither mating barriers between them, nor any notably diminished hybrid fertility except in one feature (see below).

There is a well expressed, although incompletely explored, premating barrier between *E. crameri* and *E. a. graeca*. However, no disturbances of fertilization or of hybrid development have been noticed insofar as can be distinguished from breeding problems. It should be noted here that species of the genus *Euchloe* (and *Anthocharis*) are not convenient for crosses in which many individuals are needed, because the larvae are cannabalistic feeding on both nearby eggs and later other young larvae living on the same stem. Larvae are particularly vulnerable in the moulting stage. Thus usually not more than 20 pupae result from 100 - 150 layed eggs even with the best of care. When prolonged pupal diapause is added to these problems (diapause can be lasting 2, 3 or even 6 years) the possibility of distinguishing between breeding failure and hybrid sterility, i.e. the known inability of hybrid female pupae to terminate the diapause, is further reduced. Such prolonged diapause was found more frequently when *crameri* was crossed with *simplonia* than with *graeca*. In one large *simplonia* x *graeca* brood all F_1 females eclosed without prolongation, a sign of their close genetic affinities.

Only one true hybrid character appeared in *crameri* x *a. graeca* crosses: a form of protogyny caused by the absence of the diapause in the female F_1 pupae, while all male hybrid pupae overwintered. This case resulted in desynchronization of the sexes, although in nature such females could mate with males of the second generation of *crameri* where it is bivoltine. In the R_1 of *crameri* x *graeca* females with *graeca* males bivoltinism was absent, but appeared in less than half of the females in the reciprocal backcross of hybrid males with *graeca* females. Such cases of female bivoltinism is not rare in butterflies (Federley, 1953) and was found also

in a *Iphiclides podalirius* female x *I. (p.) feisthameli* male cross, two taxa with a narrow overlap zone in the eastern Pyrenees and with highly fertile hybrids.

The chromosome number N = 31 is the same in *E. crameri, E. simplonia* and *E. ausonia graeca* as is the case in all other Anthocarini so far determined.

As in all previous *Euchloe* hybidizations, a highly, though possibly not fully, fertile F_2 generation was produced, as is usual in hybridizations below the specific level. Thus *E. crameri* and *E. ausonia graeca*, and probably also *E. simplonia*, seem to have barely reached semispecific status and to lag in their genetic differentiation far behind the pair *Pieris rapae - P. mannii*. Nevertheless, the EDf value of the *Euchloe* hybrids was nearly twice as large as that of the *Pieris* hybrids, but neither their extent nor direction is consistent with the degree of genetic reproductive incompatibility.

Pieris napi - P. bryoniae. The findings of an EDf value as low as 0.008 for *napi - bryoniae* startled taxonomists acquainted with the matter (Courtney, 1982) because it suggested that *bryoniae* is a taxon ranking below that of a subspecies. It is less than probable that this result represents the true biological distance between the two taxa. The reasons are as follows: 1)the karyotype of *napi* is a constant N=25 throughout Europe, from Moscow to the Balkans and Pyrenees and the British Isles. In *P. bryoniae,* on the other hand, N is usually 26 - 27, sometimes 25 (SE Alps) or 28, with additional 0 - 3 supernumeraries (Lorković, 1968), 2) the F_1 males of *napi* x *bryoniae* hybrids are highly, and the F_1 x F_1 matings rarely, fertile due to the mostly infertile females.

The greatest obstacle to the successful reproduction of the hybrids in nature is the disruption of diapause in the hibernating female pupae (Bowden, 1953, 1955, 1957; Petersen, 1955, 1963). In spite of their normal development, non diapausing pupae produce highly infertile females. Combined with the univoltinism of the highland *bryoniae*, this excludes the hybrid females from the reproductive process and further reduces their already low fertility. Only backcrosses of the hybrid males with parental females can thus be successful. Due to bivoltinism of the mainly lowland *napi*, the occurrence of these backcrosses is low in nature. However, the sexual isolation is not complete, and there is a complicated interaction between lowland *napi* with the rather pure highland *bryoniae* populations in overlapping areas (Petersen, l.c.). On the one hand, there are examples of intrusion of the dominant *bryoniae* gene, *Br*, into lowland populations of *napi* (Lorković, 1969; Eitschberger, 1983). On the other hand, a study of the genetic composition of the *bryoniae-napi* populations from the upper Sava valley at the extreme southeastern outskirts of the Alps suggested that the *bryoniae* form there is not hybrid, as maintained earlier, but a reproductively isolated (from *napi*) form of *bryoniae*

karyotypically identical with the Alpine form (Lorković, 1968). For further genetic information about these taxa the cited work of Bowden and Petersen should be consulted.

More recently Geiger (1984) extended his studies of enzyme similarities to other European subspecies: *thomsoni* from the British Isles, *meridionalis* Heyne & Rühl, 1895 from Italy, *adalwinda* Fruhstorfer, 1909 from Scandinavia and *P. pseudorapae balcana* from Yugoslav Macedonia. The results were similar to those of *bryoniae*. The lack of difference between *adalwinda* and *bryoniae* is not surprising since they are nearly identical. Less persuasive is the close enzytmatic similarity of *balcana* and *napi*, which are essentially reproductively isolated. The karyotype of *balcana* is like that of *bryoniae*, containing 26 - 27 bivalent and 0 - 3 tiny univalent chromosomes, and different from the *napi* karyotype. *P. pseudorapae* Verity, 1911, from Anatolia and ssp. *suffusa* from the Transcaucasus (Lorković, 1968/69) are somewhat similar to the karyotype of *napi*. The taxa predominantly contain the 25 chromosomes characteristic of *P. napi*, while the *bryoniae* karyotype is less freqently represented. The *napi* karyotype, however, is seldom found in *balcana*.

In strong contrast according to the recent enzymatic data of Geiger (1984), the North-American *napi* group taxa are sufficiently different from *napi* and from each other to be considered species. This is in agreement with Eitschberger (1983) who listed *P. venosa, P. marginalis, P. virginiensis* and *P. oleracea* as distinct species. Although definitive publication on enzymatic similarity in these taxa is not yet available, the sexual isolation of North American *P. oleracea* and European *P. napi* is very incomplete. Since F_2 and F_3 generations of the respective crosses are obtained without difficulty, the genetic-reproductive isolation seems to be even less complete (see Bowden, 1972).

As an example, a cross deisgned to explain the lack of the dark upperside wing pattern of *P. oleracea* should be mentioned. *P. oleracea* was crossed with *f. "funebris"* (Lorković, 1971) which has a heavily pigmented phenotype, recessive with respect to wild type *napi*. In the F_1 the *napi* phenotype appeared. This shows that *oleracea* contains the dominant *napi* gene for the usual *napi* melanistic pigmentation. In spite of dominance, this gene is not expressed in the phenotype of *oleracea* suggesting a recessive suppressor gene is involved. Indeed, in the F_2 all combinations of characters of *napi, "funebris"* and *oleracea* were obtained. In one of the F_3 a pure stock of homozygous *"funebris"* appeared having the wing pattern only in traces, and thus analogous in appearance to *P. oleracea*. Obviously, the recessive gene for *"funebris"* pigmentation was combined here with a recessive homozygous supressor gene. Such an extensive genetic analysis would not have been possible if *oleracea* were genetically incompatible with *P. napi*, as suggested by Eitschberger (1983) and Geiger (1984). The taxon *oleracea* is genetically more closely

related to *P. napi* than *P. bryoniae* is related to *napi*, while enzymatically *oleracea* is more distant from *napi* than from *bryoniae*—another example of sterility versus enzymatic discrepancy.

The closeness of the *oleracea-napi* relationship is reflected also in the karyotype, both taxa having the same number of chromosomes (N = 25). Thus pairing of chromosomes in meiosis is not affected.

Least but not last, three of the five North American taxa are allopatric or only slightly parapatric with narrow zones of overlap, as illustrated by the extensive material analyzed and systematized by Eitschberger (1983). Accordingly, these taxa should be classified between subspecies and species not only genetically, but also on the basis of geographic distribution.

Crosses between Forms and Subspecies

That the low fertility and viability of the interspecific hybrids, found in these investigations, do not reflect adverse breeding conditions is evidenced by breeding variants of one and the same population as well as from crosses of taxa considered to be geographically separated subspecies.

1) As an example of the first kind, during the selection of the form "*confluens*" of *Pieris rapae*, inbreeding was carried out for 10 generations. The number of offspring gradually decreased due to diminution of pairing drive in males. After the tenth generation, a male was introduced from outside. Following this inbreeding was continued for 10 more generations. Consequently, the decrease of fertility was nothing other than inbreeding depression, documented so clearly by Oliver (1981). No true genetic incompatibility or sterility has been observed.

2) For crosses at the level of morphs or forms, breeding of *P. napi napi* (Zagreb) x *P. napi* f. "*sulphurea*" (England) should be mentioned. Neither mating behavior reluctance nor infertility occurred, so that homozygous double recessive "*sulphurea funebris*" could be obtained. A F_3 generation was, of course, necessary to get sufficient numbers of progeny.

3) Among the spatially separated subspecies, *P. napi* from Zagreb was crossed with ssp. *migueli* (Eitschberger, 1983) and with the ssp. *santateresae* (Eitschberger, 1983 = *dubia* Röber, 1908). Eitschberger provided specimens of these from Spain in 1972. It was found that *P. napi santatersae* mates normally with *P. napi napi*, yielding an abundant F_1 and F_2 without of occurrence of any diapause disturbances, confirming the subspecific nature of the taxon (Eitschberger, 1983).

However, with ssp. *migueli* from Picos de Europa *napi* males do not react promptly to *migueli* females, as 10-15 s were required for *migueli* females to quiet down following introduction of the *napi* males, although

the same delay was observed also when *migueli* males and females reacted to one another. I had the impression that my laboratory was too warm and dry for ssp. *migueli* to thrive, which is in agreement with the fact that Eitschberger had better breeding success in Germany. Conversely, I had more success than Eitschberger in breeding ssp. *santateresae* from Central Spain. Pairing of both Spanish subspecies progressed without disturbance and normally developed males and females, including diapause, were produced from 5 matings.

Crosses between several other subspecies involving fewer individuals progressed normally. Thus there is no reason to suspect faulty breeding conditions as the cause of sterility found in the other Pieridae crosses reported.

Chromosome Pairing and Sterility

Hybrid sterility is usually accompanied, if not a function of, deviations of chromosome numbers of the taxa crossed. Sterility is here reflected by the failure of the homologous chromosomes to pair during the maturation process of meiosis and is easily visible in equatorial plates of meiotic divisions of the spermatocytes in the hybrid testes. One example is illustrated in Figure 3, for a *Pieris rapae* x *P. mannii* hybrid. Here, instead of 25 regularly paired chromosomes (bivalents), different numbers (25 - 34) of unpaired, univalent, smaller chromosomes can be detected. A recapitulation of data on chromosomal irregularities which are related to hybrid sterility is given in Table 3. The number of the testes and metaphase plates examined, the number of parental bivalent chromosomes found as well as bivalents of the taxa crossed, their average number, and the frequency of deviations in pairing are given.

Counting chromosomes in the testes of hybrids can be rather laborious due both to the scarcity of meiotic divisions and even more so due to the uneven chromosome plates in which some chromosomes cover and mask others using the paraffin microtome section method. In Table 3 only unequivocal chromosome numbers are given, with the exception of the single *Anthocharis eupheoides* x *A. cardamines* cross of which only a single, much inclined plate of 35 $\pm$ 3 chromosomes could be approximately estimated.

The number of chromosomes is less variable within the testes of the same individual than between different individuals of the same cross. So in 19 examined crosses of *P. rapae* females and *P. mannii* males a single plate with 43 chromosomes was found while in the reciprocal cross with *mannii* females the much lower number 25 - 34 was common. The greatest chromosomal deviation from the norm in the present hybrid series resulted from a barely fertile backcross of *P. rapae* female x (*P. mannii* female x *P. rapae* male) male. The single male examined had 40

Table 3. Chromosome number (CN) in the metaphase plate of meiotic divisions of spermatocytes of parental and F_1 testes in the hybridization of closely related Pierid taxa. In taxa with unequal parental CN the mean value is set in parentheses. The difference between P and F_1 CN indicates failures in the pairing of homologous chromosomes and is expressed as frequency.

Taxa Crossed	Number		Chromosome Number			
	Testes Examined	Plates Observed	P	F_1 Mean	F_1 Range	Frequency of Pairing Failure
1. *Pieris rapae* ♀ x (*mannii* ♀ x *rapae* ♂) ♂	1	5	25	43	40-46	.42
2. *P. rapae* ♀ x *P. napi* ♂	2	1	25	40	40	.38
3. *P. mannii* ♀ x *P. napi* ♂	6	2	25	37.5	36-39	.33
4. *P. rapae* x *P. mannii*	13	10	25	38.4	25-43	.35
5. *Anthocharis cardamines* ♀ x *Euchloe ausonia graeca* ♂	1	4	31	47.7	44-49	.35
6. *E. ausonia graeca* ♀ x *A. cardamines* ♂	1	1	31	47	47	.34
7. *A. euphenoides* ♀ x *A. cardamines* ♂	1	1	31	38	38	.18

8. *P. ergane* ♀ x *P. napi* ♂	4	16	25.3 25	27.8	25-28	.09
9. *P. napi* x *P. (napi) bryoniae*	43	60	25 25-32 (27.6)	26.5	25-28	.05
10. *P. napi* x *P. (pseudorapae) balcana*	9	9	25 25-28 (27.1)	26.1	25-28	.04
11. *P. (napi) bryoniae* x *P. (pseudorapae) balcana*	9	19	(27.6) 25-28 (27.1)	26.3	25-28	.04
12. *P. napi santateresae* ♀ x *P. napi napi* ♂	5	14	25	25.2	25-25.5	.01
13. *P. napi migueli* ♀ x *P. napi napi* ♂	5	8	25.1 25	25	25	.004

As supernumeraries are about one quarter to a half the size of other chromosomes, they were calculated as 0.25-0.50 for one chormosome and added to the whole set in proportion to their frequency. Matings without sexual signs involved reciprocal crosses.

chromosomes in one and 46 in four metaphase plates, the extreme case in which only 4 chromosomes pair instead of 25, unexpected for backcross individual, which are mostly fertile.

Although the *Anthocharis* x *Euchloe* hybrids are at the bottom of the fertility scale (Tables 1 and 2), failure of their chromosomes to pair (34 - 35) is proportionally not greater than in the *Pieris* crosses (34 - 38) which are higher in respect to fertility. This circumstance may be due to the fact that the Anthocharini have 20% more chromosomes than Pierini. They therefore probably have about 20% shorter chromosomes on the average which diminish the probability for conjugation irregularities for about the same percent. That the sterility of *Anthocharis* x *Euchloe* crosses is even greater than would be indicated by the pairing disturbances above is evidenced by the frequent total degeneration of the testes, which can be reduced to a somatic testes envelope without any trace of germinal cells. Depending upon the variable number of paired chromosomes, the final process of spermiogenesis is interrupted at different maturation levels. Sometimes testes do not contain either any sperm bundle or initial germ cells. Nevertheless, in a large fraction of adult hybrid specimens the testes contain large numbers of sperm bundles, but these sperm are no more fertile than those from testes with only a few sperm bundles.

The *Pieris napi-bryoniae-balcana* group is as yet unresolved taxonomically. Karyological definition is also not precisely defined. Not only does the different specific chromosome number between *napi* and the other taxa make the degree of pairing failures uncertain, but further problems are encountered due to the inconstant chromosome number in *bryoniae* (and *balcana*) itself, varying from N = 25 - 28. In *bryoniae* even higher numbers, 29, 30, 31 and 32, have been recorded from four individuals (Lorković, 1968). As a further complication, 1 to 3 minute univalent supernumerary chromosomes occur in a various percentage of *bryoniae* individuals. These usually lie off the equatorial plate and pass, mostly undivided, into only one daughter cell, presumably without a deleterious genetic effect (White, 1973). The supernumaries thus were neglected in the present calculations.

Eighty-nine *bryoniae* specimens from nature and 74 hybrids of F_1, F_2 and R_1 - R_4 have been karyologically examined (Lorković, 1968). In the F_1 hybrids, N = 25 and 26 were predominant (13 and 10 individuals respectively). The other numbers occurred in a small number of individuals, and 33 - 44 chromosomes were counted in a backcross individual. The known variation of hybrid fertility or sterility of *napi* x *bryoniae* crosses can be at least partly attributed to this inconstant number of *bryoniae* chromosome, thus producing the observed pairing failure of 0.05 (Table 3). However, in *napi-bryoniae* no remarkable irregularities in chromosome pairing can be cited in concordance with the finding of rather high male fertility in *napi* x *bryoniae* hybrids, but not in females which are

highly sterile. The effect is probably due to genetic factors and not chromosome mechanics.

One of the proportionally lowest values of chromosome pairing failure in crosses between two clearly defined species is the case of *Pieris ergane* x *P. napi*. This case is included although the enzyme relations are not yet known. The sterility of the hybrids of these two species is so low that even a partially fecund F_2 can be produced, while the backcrosses are normally fertile. This high fertility coincides with low failure rate of chromosome pairing, .09, falling in value between the full specific and subspecific stage. Nevertheless, *P. ergane* and *P. napi* are fully sympatric, and, although very close, both karyologically and morphologically, no worker has regarded these as other than good species.

The lowest number of irregularities of meiotic chromosome pairing occur in crosses of *Pieris napi* subspecies in which 25 pairs of chromosomes are regularly found. The cross *P. napi santateresae* x *P. napi napi* yielded a tiny supernumerary which disappeared in 5 backcrosses with ssp. *migueli*.

Unfortunately, the only fixed and prepared testes of *Euchloe simplonia* x *E. ausonia graeca* proved to be too old and without meiotic divisions. Its normal size and complement of ripe sperm is in agreement with the high fertility of F_1 individuals. It is reasonable that the meiotic divisions are normal.

Although a substantial number of karyotypes reported are not known from more than one equatorial plate, the data in general are in accordance with a decreasing trend of hybrid sterility as shown in Tables 1 and 2.

Discussion

Comparison of electrophoretic enzymatic dissimilarity and hybrid sterility in the Pieridae shows that differences between the results of both procedures are relatively much smaller between species which belong to two genera, subgenera or groups of species than among taxa at the lowest taxonomic rank, such as subspecies, semispecies or sibling species. The question here is to explain this discrepancy and to decide which of the methods is more reliable for the estimation phylogenetic relationships. If enzyme similarity is the measure of relationships, how is it possible that two entities biologically as well differentiated as *P. napi* and *bryoniae* are enzymatically almost identical; while *E. crameri* and *E. simplonia*, which are more compatible genetically, show a disproportionally high enzyme diversity, 21 times higher than in *bryoniae-napi* and nearly twice as high as the reproductively highly incompatible *rapae-mannii* pair?

One reason for the discrepancy may be that the taxonomic estimation of divergence at low levels is not sufficiently confined, i.e. the taxonomy at

the lowest levels is not precisely enough graduated to produce unequivocal categories. One approach to correct this weakness in taxonomy would be broadening the biological species concept to cover partially hybridizing taxa, combined with a stronger emphasis on spatial and ecological differentiation. More than twenty years ago Ehrlich (1961) emphasized that the biological species concept was outliving its usefulness. Nevertheless, in 1982 Ehrlich and Murphy explained "that sympatric synchronic populations that do not interbreed should be considered to belong to separate species" or "if it were certain that successful interbreeding is not possible. . .would we elevate. . .to specific status".* Obviously the gap between the biological species concept and the nonbiological is not so serious as seems at first glance, and that it is not necessary to create more confusion where enough is already present. Although the incompleted stage of speciation does not have a generally accepted taxonomic term (semi species or similar), such a category is indispensable in evolutionary biology as well as in taxonomy itself. Clearly it is incomplete speciation that is the heart of the nearly hundred years of argument over the *napi-bryoniae* problem. This situation contrasts with that of the remarkable specific stability in the genus *Leptidea*, of which *L. morsei* cannot be distinguished over its entire palearctic range from Far East to eastern Europe, including its invariable and usual karyotype of N = 54.

Discrepancy among taxonomist's views also arises from unequal and unevenly developed isolating mechanisms found during the initial phases of speciation. For example, *Euchloe crameri* appears sexually separated from *E. simplonia*, but there are no other reproductive disturbances characteristic of higher rank of speciation. On the other hand, these two taxa behave like two subspecies in being almost completely allopatric. Therefore, as usual in such situations, the question arises as to how much the differentiation is due to an intrinsic genetic barrier and how much is due to geographic and ecological displacement.

In evolutionary processes, isolating mechanisms frequently appear independent from one another and at unequal rates. Hybrid incompatibility may arise between separate taxa, reflected in the infertility of the female sex, before the aquisition of a premating barrier. Such a situation is found for *Pieris napi* (Europe) and *P. dulcinea psuedonapi* (Japan) (unpublished), or in the reverse sequence, as already described for the *Euchloe* pair.

A serious obstacle to the use of enzymatic differences in taxonomy is the inequality of the EDf levels characteristic of species in different systematic groups. To mention only a few examples in Lepidoptera, Racheli, et al. (1984) found average EDf values between 0.15 and 0.11 in four

*The excellent paper of Murphy and Ehrlich (1984) appears too late to be incorporated in this paper. I agree with several good points made by these authors concerning the future of taxonomic work with the Lepidoptera.

among six south european geographic subspecies of *Parnassius apollo*, while the remaining two had 0.09 and 0.08. If the taxonomic levels of this group were to be estimated according to the criteria used for *Pieris rapae* and *P. mannii* (EDf = 0.10) then most of these subspecies would have to be classified as species. In the case of *P. apollo*, ssp. *hispanica*, perhaps also ssp. *pumilio*, such a conclusion would be valid if hybrid sterility were found experimentally, as enzyme criteria alone would be insufficient. The claim that at least one, if not all three, of the *P. apollo* subspecies should be considered species can be decided only by crossings. Then enzymes can be viewed as a stimulus for crossing experiments. In any case, it would be rewarding to perform hybridization experiments between some well differentiated subspecies to know finally how clearly they are reproductively differentiated.

Such uncertainties have been known for some time. Originally it was considered that greater EDf differences exist between taxa which are above the level of genus, but Bullini, et al (1981) emphasized that no rule exists and the distance between failies can be very large although in some cases not larger than between species.

On the other hand, enzyme differentiation does not appear to be a causative factor of reproductive separation, but rather an effect of isolation. Their evolutionary significance thus appears to be similar to that of other genes which influence small morphologic changes, as for example the specific genitalia which mostly do not impede hybridization. Thus the genitalia are much less efficient than premating olfactory obstacles. Natural copulation between *C. hyale* and *C. australis* is completely impossible, but mating ensues in reciprocal directions as soon as the olfactory barrier has been artificially removed (Lorković, 1953 and unpublished data). As with other characters, those enzymes selected for analysis may also well affect conclusions of relationships.

Speciation appears to be a capricious event and is not only a simple quantitative process, solely due to an accumulation of genetic differences. Speciation is clearly less dependent or independent of merely allelic alterations and substitutions. Ayala (1975), Oliver (1978), Bullini and Sbordoni (1980), discussing genetic differentiation and hybrid incompatibility, all point out that electrophoresis of enzymes cannot reveal more than 30% of all genetic differences, while other differences, such as those of regulatory genes are not accessible to this technique. Regulatory genes are responsible for the coordination of embryonic development, growth and differentiation, all of which are crucial for the success of specific hybridization. Chromosomal rearrangement may also have an important role here in agreement with the absence of regular chromosomal pairing in hybrids at the species level described in this paper.

Enzyme electrophoresis is without question of great significance to the

study of genetic differentiation of populations, but it would appear that the method is of most value at the micro evolutionary level and not as an infallible device for delimiting taxa since genetic reproductive isolation does not always coincide with any given amount of enzyme diversity. Since many extensive discussions of this problem have been made elsewhere, the results of the present contribution mainly serve to issue caution for the careful systematic interpretation of enzyme electrophoresis.

Acknowledgments. The author is most grateful to R. H. T. Mattoni for general help with the manuscript, and Drasko Serman, Institut for Biology, Medical Faculty, Zagreb; Hervoje Lorković, Abt. Allgemeine Physiologie, University of Ulm, Germany, for the helpful criticism, remarks and improvement of the English text. Without the many years of generous cooperation with Sydney Bowden and his provision of living specimens, this paper would not have been possible.

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